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(54) **Immunoassay for determinatin of psa-act**

(57) A method for determining PSA-ACT in a blood sample by two-site immunometric assay using a labeled antibody reagent and a capture antibody reagent which is immobilized or immobilizable to microparticles. Resulting immobilized immunocomplexes are washed, preferably multiples times and preferably by an automated analyzer, and the labeled antibody reagent measured in the washed microparticles. Measurement of PSA-ACT provides a method for aiding in the diagnosis and monitoring of prostate cancer that is highly sensitive and specific, and eliminates the need for a significant number of patients to undergo unnecessary prostate biopsy.

Fig. 2

A

B PSA-ACT Recovery from 5 Mixtures at 10ng/mL
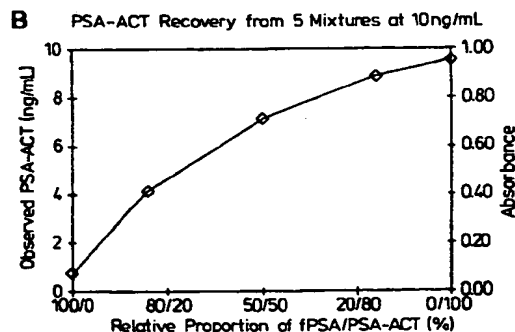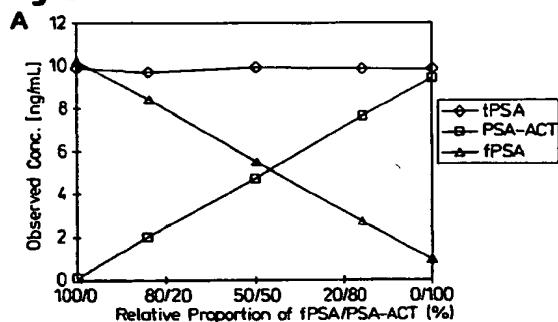
EP 0 895 085 A1

Description

BACKGROUND OF THE INVENTION

[0001]   The present invention relates to the accurate and specific determination of the complex of prostate specific antigen (PSA) and alpha-1-antichymotrypsin (ACT) in a blood sample by a two-site immunometric assay.

[0002]   Human prostate specific antigen (PSA) is a glycoprotein of approximately 33,000 daltons with high amino acid homology to the human kallikrein family (1,2) and has been shown to be a serine protease with trypsin and chymotrypsin-like activity (3,4,5). PSA is secreted by epithelial cells of the prostate gland and is one of the major proteins found in seminal fluid (6). Following the discovery that the concentration of PSA increases in the serum of patients with prostate cancer, numerous reports have established this protein as an important and clinically useful biomarker for the management of prostate cancer patients (7,8,9,10). Recent efforts have focused on the use of serum PSA testing for early detection of prostate cancer in asymptomatic men. In fact, the American Cancer Society and the American Urological Society have recently recommended that all men over the age of 50 be screened annually using serum PSA in conjunction with digital rectal examination (DRE) (11).

[0003]   The clinical value of early detection of prostate cancer remains controversial for several reasons. First, it is unclear whether treatment of prostate cancer at early stages will improve survival in the affected population. Clinical trials designed to address this issue are currently underway. Second, a clinical trial recently measured the effectiveness of serum PSA measurements in conjunction with digital rectal examination (DRE) for early detection of prostate cancer in men over 50 years of age (12). Of the 1060 patients who had either an abnormal DRE or an elevated PSA test; only 22% had prostate cancer. These data demonstrate that 70-80% of all prostate biopsies are performed on men who do not have cancer. Since 30-50% of men over the age of 50 have evidence of prostate cancer on autopsy, the number of unnecessary prostate biopsies triggered by elevated PSA assays could be very high. This has consequences both in increased medical costs and increased morbidity associated with the biopsy procedure.

[0004]   Several laboratories have shown independently that PSA forms complexes with protease inhibitors such as α_1-antichymotrypsin (ACT), α_2-macroglobulin, and α_1-antitrypsin (13-19). PSA in complex with ACT or α_1-antitrypsin or in free, uncomplexed form is detectable in serum by immunoassay techniques. Indeed, the majority of immunoreactive PSA in serum is complexed with ACT, and a significant correlation has been established between the proportion of PSA bound to ACT and total serum PSA concentration (13). PSA bound to α_2-macroglobulin, however, is not measurable in serum due to steric hindrance of antibody binding to PSA following complexation with this protease. In early work, PSA-ACT levels and the proportion of PSA-ACT to total PSA were suggested to be of use in prostate cancer diagnosis (13,15,16,17), however, for a variety of reasons (some of which are discussed below) it has been difficult to draw conclusions on the clinical utility of serum measurement of PSA-ACT.

[0005]   Lilja, Stenman, and coworkers published in 1991 that serum PSA exists in free form and in complexes with ACT and α_1-antitrypsin (13,18). In subsequent work, Stenman et al. demonstrated that measurement of PSA-ACT in association with measurement of free plus complexed PSA (termed total PSA, although PSA complexed with α_2-macroglobulin is not measured by conventional PSA assays) may improve discrimination between men with prostate cancer and those with benign prostate disease such as benign prostatic hypertrophy (BPH). However, the accurate measurement of PSA-ACT complexes has not been attainable due to technical problems in accurate measurement of the complex. Stenman et al. found that the correlation of PSA-ACT values with total PSA measurement was not good at the low end and the y intercept was elevated indicating over-recovery of complexed PSA (13 and U.S. Patent No. 5,501,983). Indeed, they found that for most patients tested, the concentration of PSA-ACT was higher than for total PSA (U.S. Patent No. 5,501,983). Subsequent correlation analysis for complexed and free PSA showed a slope of 1.12 indicating over-recovery of the PSA-ACT complex (16). Pettersson et al. addressed this over-recovery when they found elevated PSA-ACT values in female sera (20). While the addition of heparin reduced the incidence of false positive values in female serum, more recent attempts to measure PSA-ACT complexes in patients with prostate cancer and BPH continue to show significant over-recovery of complexes (21).

[0006]   Because of the difficulties encountered in the measurement of PSA-ACT complexes, attention in the literature turned to the measurement of free, uncomplexed PSA in conjunction with measurement of total PSA. It is now clear that improved specificity is needed when total PSA values range from about 4-10 ng/mL. When serum total PSA is <4.0 ng/mL, the risk of prostate cancer is low; similarly, when total PSA is >10 ng/mL, the risk of prostate cancer is >50% and prostate biopsy is indicated. Within the diagnostic gray zone (generally between 2-20 ng/mL, more commonly between 4-10 ng/mL) the risk of cancer is high, but the rate of false positives is also high. The retrospective application of a ratio of free PSA/total PSA has shown that the specificity of total PSA in the 4-10 ng/mL gray zone could be improved from approximately 50-60% to 70-80% (22-26). This improved specificity could result in a 20-30% decrease in unnecessary biopsies. PCT WO 96-26441 and WO 97-12245 similarly describe the

use of the free PSA/total PSA ratio to improve discrimination between BPH and cancer, respectively, in patients with total PSA levels between 2.5 and 20 ng/mL.

[0007] The measurement of free PSA has technical difficulties of its own, however. First, within the diagnostic gray zone, the proportion of free PSA is typically quite low, in the 5-30% range. A successful free PSA assay must, therefore, measure accurately in the range of 0.2-3.0 ng/mL. Also, the concentration of free PSA is not significantly different in patients with BPH and cancer, and the ratio of free PSA/total PSA decreases due to an increase in the proportion of PSA complexed to ACT. In addition, free PSA is not stable in serum and levels of free PSA have been known to decrease over time, presumably due to complexation with α_2-macroglobulin.

[0008] In the meantime, there has been further acknowledgment of the problems associated with the accurate measurement of PSA-ACT in blood, coupled with attempts to overcome such problems. In 1994, workers at Hybritech reported the development of a sandwich immunoassay for PSA-ACT employing anti-PSA and anti-ACT antibodies. They concluded that the measured PSA-ACT values failed to demonstrate improved clinical specificity in the diagnosis of prostate cancer (27). Later, this group jointly with workers at the Johns Hopkins Medical Institutions reported the finding that the anti-PSA/anti-ACT sandwich immunoassay method suffers from significant non-specific binding and over recovery of PSA-ACT. Unless resolved, they concluded that these problems rendered the measurement of PSA-ACT clinically meaningless (28). Subsequently, this joint group reported having overcome the non-specific binding problem through the development of a sandwich immunoassay for PSA-ACT based on a monoclonal antibody specific for PSA-ACT complex (29, 30). However, their clinical studies failed to show any improvement in specificity for prostate cancer by measuring PSA-ACT complex alone compared with measurement of total PSA or with a calculated ratio of PSA-ACT to total PSA (29). Other approaches to overcoming the problems associated with PSA-ACT measurement have been reported, including the use of blocking agents (31).

[0009] It remains unclear why the proportion of PSA complexed to ACT increases in patients with prostate cancer, but it may be related to the observation that antibodies to ACT do not stain prostatic epithelium from BPH patients and mRNA transcripts are not found in such tissue. In contrast, anti-ACT immunoreactivity and mRNA synthesis are detected in prostatic epithelium from patients with prostate cancer (32). These results suggest that in prostate tumors, PSA may complex in situ with ACT prior to release into serum. An alternative mechanism may involve the access of active PSA to the blood stream. Free PSA found in serum from healthy men is proteolytically cleaved and enzymatically inac-

tive. Tumors, however, synthesize angiogenic factors which lead to increased vascularization of tumor tissues. It may be that in tumors, a larger proportion of enzymatically active PSA gains access to the blood stream. This active PSA would be expected to complex with protease inhibitors such as ACT leading to a higher proportion of PSA-ACT complex in serum from prostate cancer patients.

[0010] Accordingly, there is a need for an accurate method of determining complexed PSA and to assess the clinical significance of blood levels of complexed PSA relative to screening of male patients for prostate cancer.

[0011] EP 635,575 describes the preparation of monoclonal antibodies that bind to free PSA but not PSA-ACT.

[0012] PCT WO 95/18381 relates to a monoclonal/polyclonal immunometric assay method for the determination of PSA which is rendered capable of providing an equimolar response to free and complexed PSA by the addition of antibody that binds to free PSA but not complexed PSA.

[0013] U.S. Patent Application Serial No. 08/595,155 and Zhou Z, Ng PC, Very DL, Allard WJ, Yeung KK, J. Clin. Lab. Anal. (1996), 10:155-159, describe a method for preparing a monoclonal antibody that provides an equimolar response to free and complexed PSA in a monoclonal/polyclonal immunometric assay. The described monoclonal antibody has the unique property of binding to PSA to render PSA substantially incapable of binding with antibodies that bind free PSA but not complexed PSA.

[0014] Published Japanese Patent Document 62-46263 describes a sandwich immunoassay method for the determination of PSA in complex with protease inhibitor.

[0015] Published German Patent Application 4,322,342 describes a method for measuring both total PSA and PSA-ACT in a single assay for the purpose of providing values for calculation of the ratio of PSA-ACT to total PSA.

[0016] Chichibu et al, in the Journal of Medicine and Pharmaceutical Science (Japan, 1996) 36(3): 477-483, describe a sandwich immunoassay for PSA-ACT employing anti-PSA bound to a bead and enzyme-labeled anti-ACT. Data establishing the ability to accurately measure PSA-ACT in a blood sample is lacking.

SUMMARY OF THE INVENTION

[0017] It has now been found that the accurate and specific determination of PSA-ACT in a blood sample can be obtained by a two-site immunometric assay in which the capture antibody is immobilized to microparticles (e.g., magnetically responsive microparticles). In general terms, the present method comprises the steps of (a) forming a reaction mixture containing immobilized and labeled immunocomplexes of PSA-ACT from the

blood sample bound to a first antibody reagent specific for binding to PSA and to a second antibody reagent specific for binding to ACT, wherein one of said first and second antibody reagents is immobilized to microparticles ("capture antibody"), and the other of said antibody reagents is labeled ("detection antibody"), (b) separating the microparticles comprising said immobilized immunocomplexes from the remainder of said reaction mixture, (c) washing the separated microparticles, preferably at least twice, and (d) measuring the labeled antibody reagent in the washed microparticles. Preferably, the wash and separation steps are performed by an automated analyzer. Also, preferably, the first and second antibody reagents comprise monoclonal antibodies. The reaction mixture containing immobilized and labeled immunocomplexes of PSA-ACT is usually formed by mixing the blood sample with said first and second antibody reagents, wherein one of said first and second antibody reagents is immobilized, or capable of being immobilized, to said microparticles ("capture antibody"), and the other of said antibody reagents is labeled, or capable of being labeled ("detection antibody"), e.g., with an enzyme or chemiluminescent compound. In a particularly preferred method, the capture antibody is modified with a ligand and is immobilized by contact with a binding partner for the ligand fixed to said microparticles.

[0018] The present method provides specific and accurate detection of PSA-ACT complexes. As demonstrated in the examples which follow, the quantitation of PSA-ACT has been shown to be accurate because the sum of the measured values for free PSA plus PSA-ACT is equivalent to the measured value for total PSA. The difficulties reported in the literature in measuring PSA-ACT using manual formats, usually based on microtiter plate separation, are overcome by the use of a microparticle-based separation, preferably involving multiple washes, and preferably being performed on an automated analyzer. The present method has also been shown by spiked recovery experiments to accurately measure PSA-ACT both in a buffered solution and in serum. In addition, testing of serum samples from healthy men age 50 years or older, patients with BPH, and patients with prostate cancer has shown that the present method has clinical utility in the discrimination of men with and without prostate cancer. The upper limit of normal of the PSA-ACT assay was established to provide equivalent sensitivity to that of the prior art total PSA assays. Using this upper limit of normal (3.75 ng/mL), the specificity of the PSA-ACT assay for the detection of CaP in males whose total PSA levels are within the prior recognized diagnostic gray zone (e.g., between about 4-10 ng/mL) has been found to be comparable to that of recently developed methods based on the calculation of an fPSA/tPSA ratio.

[0019] The present method therefore provides a particularly useful aid in the detection of CaP in male human patients. Significantly, the present method aids in the decision whether to recommend that a male patient undergo costly and discomforting prostate biopsy. In these regards, the present method has the clear advantage of requiring the performance of only a single assay rather than dual assays as required in order to calculate an fPSA/tPSA ratio.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020]

Figs. 1A and 1B are graphs showing standard curves for PSA-ACT using the PSA-ACT microparticle immunoassay and microplate-based ELISA PSA-ACT sandwich immunoassay, respectively.

Figs. 2A and 2B are graphs showing the specificities of various PSA immunoassays. Samples contained approximately 10 ng/mL total PSA with varying proportions of free and PSA-ACT. In Fig. 2A it is shown that the PSA-ACT microparticle immunoassay measures PSA-ACT specifically and accurately. In addition, tPSA assay measures PSA and PSA-ACT with an equimolar response, and the fPSA assay measures free PSA specifically. The graph shown in Fig. 2B demonstrates the recovery of PSA-ACT in a microplate-based ELISA PSA-ACT immunoassay.

Fig. 3 is a graph showing the specificity of the PSA-ACT microparticle immunoassay described in the Examples. Samples containing fPSA at the concentrations of 0, 2, 10, 25, 50 and 100 ng/mL in the MES buffer containing 6% BSA were tested with a fPSA assay and the PSA-ACT assay. The data demonstrate that almost no free PSA was measured by the PSA-ACT microparticle-based immunoassay, but fPSA was fully quantitated by the fPSA assay.

Fig. 4 is a table of data showing that the PSA-ACT microparticle-based assay accurately measures PSA-ACT spiked into serum.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0021] As used herein, the following terms shall have the indicated meanings:

PSA shall mean prostate specific antigen.

tPSA or total PSA shall mean the total amount of immunologically determinable PSA in a blood sample, that is, PSA in complexed or free forms that are capable of responding to measurement by conventional immunoassays. Based on current knowledge, it is understood that blood PSA that is complexed

with certain protease inhibitors (including ACT, α_1-antitrypsin, and inter-α trypsin inhibitor) is immunologically determinable, whereas PSA is not determinable when complexed with such other protease inhibitors as α_2-macroglobulin.

fPSA or free PSA shall mean PSA in its free, uncomplexed form.

PSA-ACT shall mean PSA complexed with ACT.

[0022]   Antibody shall mean whole immunoglobulin, e.g., IgG or IgM, or an immunoglobulin fragment comprising an antibody binding site, e.g., Fab, Fab', and F(ab')$_2$ fragments, or aggregates thereof.

[0023]   The present invention provides means for determining PSA-ACT in a blood sample by a two-site microparticle-based immunometric assay. It will be evident to one of ordinary skill in the art that a variety of two-site immunometric formats and microparticles can be used to measure PSA-ACT. In general, two antibodies are employed in a two-site immunometric assay method (sometimes referred to as a "sandwich" method). One of the antibodies is labeled or labelable (sometimes referred to as the "detection antibody") and the other is immobilized or immobilizable (sometimes referred to as the "capture antibody"). As is known in the art, the capture and detection antibodies can be contacted simultaneously or sequentially with the test sample. Sequential methods can be accomplished by incubating the capture antibody with the sample, and adding the labeled antibody a predetermined time thereafter (sometimes referred to as the "forward' method); or the detection antibody can be incubated with the sample first and then the labeled antibody added (sometimes referred to as the "reverse" method). After the necessary incubation(s) have occurred, to complete the assay, the capture antibody is separated from the liquid test mixture, and the label is measured in at least a portion of at least one of the separated capture antibody phase or the remainder of the liquid test mixture, normally the former since it comprises PSA bound by ("sandwiched" between) the capture and detection antibodies.

[0024]   In typical two-site immunometric assays for PSA, one or both of the capture and detection antibodies are monoclonal antibodies. The label used in the detection antibody can be selected from any of those known conventionally in the art. Commonly, the label is an enzyme or a chemiluminescent moiety, but can also be a radioactive isotope, a fluorophor, a detectable ligand (e.g., detectable by a secondary binding by a labeled binding partner for the ligand), and the like. The important property of the capture antibody is that it provides a means for being separated from the remainder of the test mixture. Accordingly, as is understood in the art, the capture antibody can be introduced to the assay in an already immobilized or insoluble form, or can be in a immobilizable form, that is a form which enables immobilization to be accomplished subsequent to introduction of the capture antibody to the assay. An example of an immobilizable capture antibody is antibody which has been chemically modified with a ligand moiety, e.g., a hapten, biotin, or the like, and which can thus be subsequently immobilized by contact with an immobilized form of a binding partner for the ligand, e.g., an antibody, avidin, or the like.

[0025]   In accordance with the present invention, it has been found that the inaccuracies in the prior art methods for determining PSA-ACT are overcome by the use of microparticles as the means for immobilization of the capture antibody (either by direct immobilization of th capture antibody to microparticles or by indirect immobilization through the use of ligand-modified antibody with addition of microparticle-borne binding partner). As is well-known in the art, immobilization can be accomplished by a variety of means, such as covalent or non-covalent attachment. Moreover, it is preferred to subject the microparticles carrying the immobilized immuno-complexes of PSA-ACT bound to the capture and detection antibodies to multiple washings, e.g., at least 2 or more, in order to assure removal of background interferences. After separating the microparticles from the remainder of the reaction mixture, they are mixed with a first wash liquid, e.g., buffer, separated from the first wash liquid, mixed with a second wash liquid, e.g., further buffer, separated again, and so forth for the desired number of washes. The wash and separation steps are most preferably performed by an automated analyzer in order to obtain consistency of washing.

[0026]   The above-described immunoassay methods and formats are intended to be exemplary and are not limiting since, in general, it will be understood that any two-site microparticle-based immunoassay method or format can be used in the present invention.

[0027]   A wide variety of microparticles can be used in the present invention. In general, the microparticles will be of limited size in order to promote highly efficient washing and thereby removal of potentially interfering amounts of nonspecifically bound ACT from the blood sample. Typically, the microparticles will have a mean diameter of between about 0.01 microns and about 100 microns, more usually between about 1 micron and about 50 microns. The microparticles can be composed of a wide variety of natural and/or synthetic materials and can be prepared in a wide variety of ways. Although they may be of any particular shape, essentially spherical microparticles are typically known and used in immunoassay applications. Commonly, the microparticles are in the form of latexes, colloids, hydratable gel particles, and the like. For example, a wide variety of latex microparticles are available commercially, such as from Bangs Laboratories, Carmel, Indiana, USA or Dow Chemical Company, Midland, Michigan, USA. Such latex microparticles are typically composed of such polymers as polystyrene, butadiene styrenes, ployethyl-

methacrylate, polyvinyl acetate, polyvinylpyridine, polybutyleneterephthalate, and functional derivatives thereof, e.g., bearing such groups as aldehyde, carboxy, amino, hydroxyl, or hydrazide, which serve as sites of chemical coupling of a desired antibody reagent.

[0028] Particular advantage can be taken by the use of microparticles which are magnetically responsive, e.g., , ferromagnetic or paramagnetic microparticles. Magnetically responsive microparticles will comprise a metal (such as iron, nickel or cobalt), a metal alloy (such as metallic alloys of aluminum, nickel or cobalt), and/or a metal oxide (such as Fe_3O_4 or Fe_2O_3). In preferred form, such magnetically responsive microparticles also comprise a nonmagnetic polymeric matrix or coating. Examples of useful magnetically responsive microparticles are described in U.S. Patent Nos. 4,177,253; 4,661,408; 5,206,159; 5,320,944; 5,541,072; and 5,567,326. The polymer particles containing iron oxide granules described in U.S. Pat. No. 5,206,159 are particularly preferred.

[0029] The anti-PSA and anti-ACT antibodies employed in the present method can be obtained by any conventional means, including both polyclonal antibodies obtained by harvesting antiserum from an appropriately immunized host animal and monoclonal antibodies, which are preferred, obtained by somatic cell hybridization. Somatic cell hybridization is now a well-known methodology and can be applied to the present invention in all of its variations as appropriate and desired. In general, one prepares a population of hybridomas by fusion of myeloma cells with lymphocyte cells taken from an animal that has been immunized against the analyte. Immunization of the host animal, as used herein, implies that the animal's immune system has been challenged to produce antibodies that will bind to one or more epitopes on the analyte of interest. It will be evident to the skilled worker in the art that such result can be obtained in any number of ways, including, without limitation, administration of the native analyte, synthetic peptide immunogen, tranfectant cells which express epitopes of the analyte on their surface, or the like, to the bloodstream of the host animal. Similarly, production and harvesting of monoclonal antibodies from cloned hybridoma cell lines are within the ordinary skill in the art, and in general any known method can be used in practicing the present invention.

[0030] The reagents and other assay components necessary for practice of the above-described particularly preferred method for determining PSA-ACT are conveniently provided in the form of a test kit, that is, a packaged collection or combination as appropriate for the needs of the user and any analytical instrumentation involved. Minimally, the test kit will comprise the aforesaid first and second antibodies.

[0031] PSA-ACT blood values in male patients provide substantial clinical significance in comparison to the prior art tPSA values and fPSA/tPSA ratio values. Specifically, a study was conducted using serum sam-ples from 226 patients including 53 patients with CaP, 75 patients with BPH, and 88 healthy male controls over the age of 50 years. In this study, measurement of PSA-ACT was found to give equivalent sensitivity for detection of CaP as compared with measurement of tPSA (85% v. 88%, respectively). For all patients tested, the specificity in the normal and BPH populations was also comparable for PSA-ACT compared to the fPSA/tPSA ratio. The finding that the sensitivity and specificity of the tPSA assay used in conjunction with a fPSA/tPSA ratio is equivalent to that of the PSA-ACT alone also held true when the patient population was stratified into the diagnostic gray zone. The precise range of the diagnostic gray zone has not been defined, but at all ranges compared in this study, the sensitivity and specificity of the PSA-ACT assay was comparable to that obtained using both total and free PSA assays. These data demonstrate that a single test, PSA-ACT, can detect prostate cancer as efficiently as total PSA, and, in addition, has the improved specificity that has been shown to be obtainable using two assays, fPSA and tPSA.

[0032] In addition to the above-discussed use in the detection of prostate cancer, the measurement of PSA-ACT will be useful in the monitoring of the course of disease in patients who have been diagnosed with prostate cancer, particularly after having received first line therapy for prostate cancer. Longitudinal monitoring of such patients by measurement of tPSA has been proven to be useful in the early detection of recurrent prostate cancer. PSA-ACT is understood to be the cancer-specific form of PSA and is the form that would be expected to increase in serum as cryptic cancer cells establish distant metastic sites and grow. Accordingly, changes in PSA-ACT blood levels over time will correlate with changes in disease status, and particularly, increasing PSA-ACT blood levels after therapy will indicate recurrence of disease.

[0033] The present invention will now be illustrated, but is not intended to be limited by, the following examples.

EXAMPLES

MATERIALS AND METHODS

Antibodies and Antigens

[0034] Antibodies used in this study include the following: MM1, a monoclonal antibody specific for PSA which recognizes both free and complexed PSA (substantially similar antibodies are those produced by hybridoma cell lines 346.7.4 and 346.7.26 deposited with the American Type Culture Collection, Rockville, Maryland, USA, on April 10, 1997, and assigned deposit numbers HB-12338 and 12337, respectively); and MP2, an affinity purified polyclonal antibody to PSA derived from goats. A free PSA-specific monoclonal antibody, PSA19, and two ACT-specific monoclonal antibodies, ACT 53 (Lot #

6306) and ACT 68 (Lot # 6268), were purchased from CanAg Diagnostics AB (Gothenburg, Sweden). These antibodies were purified using Protein-A affinity chromatography, and stored in TRIS-HCl buffer, pH 7.8 containing 0.9% sodium chloride.

[0035] Antigens used in these studies include free PSA (Lot #, 751464) and PSA-ACT complex (Lot #, 145564) purchased from Scripps Laboratories (San Diego, California, USA). Free PSA was purified from human seminal fluid with 98% purity as determined by SDS-PAGE and enzyme immunoassays and stored in 10 mM TRIS buffer, pH 8.0, containing 0.1% sodium azide. ACT was purified from human plasma to approximately 96% purity. PSA-ACT complex was stored in 10 mM sodium acetate buffer, pH 5.6, containing 150 mM sodium chloride and 0.1% sodium azide. Free PSA and PSA-ACT antigens were used for the preparation of the tPSA, fPSA, and PSA-ACT calibrators.

Buffers and Solutions

[0036] A buffer for use with antibody conjugates was prepared using 100 mM TRIS-HCl, pH 7.4, with 5% heat-inactivated normal goat serum (Biocell Laboratories, Carson, California, USA). 25 mM TRIS-HCl buffer, pH 7.4, containing 111 mM sodium chloride and 6% BSA was used to prepare calibrators for the Bayer Immuno 1™ total PSA and free PSA assays (Bayer Corporation, Business Group Diagnostics, Tarrytown, New York, USA). MES buffer (50 mM), pH 5.8, containing 111 mM sodium chloride and 6% BSA was used to prepare calibrators for the PSA-ACT assay.

Calibrators and Controls

[0037] PSA-ACT calibrators were prepared at six concentrations: 0, 2, 10, 25, 50, and 100 ng/mL, using the 50 mM MES buffer. These calibrators were used in both the Bayer Immuno 1 PSA-ACT assay and the manual PSA-ACT ELISA assay (both as described below). Purified free PSA from human seminal fluid was suspended at the same six concentrations in the TRIS buffer described above for use in the total and free PSA assays.

[0038] Two sets of PSA-ACT controls were prepared. One set contained two concentrations of PSA at 4 and 25 ng/mL using 90% PSA-ACT and 10% free PSA using the TRIS-HCl buffer as a diluent. A second set of controls was prepared using three concentrations of PSA-ACT of 3.5 , 15 and 75 ng/mL using the 50 mM MES buffer. These two control preparations were used in the Bayer Immuno 1 PSA-ACT assay and the manual PSA-ACT ELISA. A commercial tri-level control product was obtained from BioRad Laboratories, California, USA. A control product using human serum spiked with free PSA to a final concentration of 4 ng/ml was prepared at Bayer Corporation. The latter two materials were used as controls for the Bayer Immuno 1 tPSA and fPSA assays.

Serum Samples

[0039] A total of 216 serum samples from 53 patients with prostate cancer and from 163 BPH patients and normal males age 50 years or older were obtained from BioClinical Partners (Franklin, Massachusetts, USA). The diagnoses of prostate cancer and BPH were confirmed by histology, and men were considered normal with regard to prostate disease on the basis of a normal digital rectal examination.

The Bayer Immuno 1™ PSA Assay

[0040] The Bayer Immuno 1 PSA assay is a commercially available sandwich assay which uses a monoclonal antibody for capture and polyclonal antibodies for detection of PSA. The monoclonal anti-PSA antibody is conjugated to fluorescein (R1, 1.5 mg/mL) and the affinity-purified polyclonal antiserum is conjugated to alkaline phosphatase (R2, 6.5 mg/mL). Solutions of the two antibodies, at 65 ml/test, are incubated with specimen to form an immunocomplex (R1-PSA-R2) which is then captured by magnetic particles (10 ml/test) coated with anti-fluorescein monoclonal antibodies. The particle bound immunocomplexes are washed and then 300 ml/test of 23 mM p-nitrophenyl phosphate are added and the rate of its conversion by bound alkaline phosphatase to p-nitrophenolate is measured. Calibration of the Bayer Immuno 1 analyzer is performed using the Bayer Immuno 1 SET point® PSA calibrators, prepared from free PSA at concentrations of 0, 2, 10, 25, 50 and 100 ng/mL. A cubic-through-zero fitting algorithm is used to generate a standard calibration curve. Operation of the Bayer Immuno 1 analyzer is performed according to the manufacturer's instructions (Bayer Corporation, Tarrytown, New York, USA).

Microparticle-Based Assay for PSA-ACT

[0041] The Bayer Immuno 1 PSA-ACT assay format is the same as that of the Bayer Immuno 1 tPSA Assay except for the following changes: (1) a monoclonal antibody specific for ACT, ACT 53, is used for detection at 2 µg/mL; (2) PSA-ACT is used as the calibrator and control antigen with the 50 mM MES buffer, pH 5.8, as a base; and (3) a two-wash protocol is used such that antigen is first incubated with capture antibody, the resulting complex is washed to remove unbound antigen and other serum components, and then the detection antibody is added. This is in contrast to the format used in the Bayer Immuno 1 tPSA assay where antigen is incubated simultaneously with the capture and detection antibodies.

Automated Assay for free PSA

[0042]   The protocol used for the Bayer Immuno 1 fPSA assay is the same as that described above for the Bayer Immuno 1 tPSA assay except the following changes: (1) monoclonal antibody, PSA19 specific for fPSA, is conjugated to fluorescein for capture; (2) the concentrations of R1 and R2 used are 2.5 µg /mL and 6.15 µg/mL, respectively; and (3) the sample volume is increased from 20 µl to 35 µl per test.

Manual Microplate ELISA Method for PSA-ACT

[0043]   Immulon 1® microtiter plates (Dynatech Laboratories, Chantilly, Virginia, USA) were coated with the MM1 capture antibody using 100 µl/well at 1.5 µg/mL in 100 mM sodium carbonate buffer, pH 9.6. The plates were incubated at room temp for 1.5 hr. Unreacted sites were quenched with 300 µl/well of TRIS, pH 7.4 containing 6% BSA, and incubated overnight at 4°C. A 100 µl volume of either PSA-ACT calibrators, controls, or patient samples was added and incubated at room temperature for 1.5 hours. After 5 washes with TRIS buffered saline using an Ultrawash II Microplate Washer (Dynatech Laboratories, Chantilly, Virginia USA), ALP-ACT #53 detection antibody (100 µl/well) in GS5 buffer was added and incubated for 1.5 hours at RT. After 5 more washes 100 µl of p-nitrophenyl phosphate substrate solution (1 mg/mL) in diethanolamine (Pierce Chemicals, Rockford, Illinois, USA) was added and incubated at room temperature. The immunoreaction was stopped with the addition of 100 µl/well 1N sodium hydroxide. Absorbance of each well was read at 405-490 nm on Thermo-Max Microplate Reader (Molecular Devices, Menlo Park, California, USA). To assure accurate quantitation of PSA-ACT, a calibration curve was run in each microplate. The absorbance was converted to analyte concentration using a cubic fit through zero algorithm. All samples were run in duplicate.

RESULTS AND DISCUSSION

Selection of Antibodies for the measurement of PSA-ACT

[0044]   Two monoclonal antibodies, ACT 53 and ACT 68, specific for ACT, were selected for analysis as detection antibodies. ACT 53 was used at 2 µg/mL and ACT 68 was used at 4 µg/mL in combination with the capture antibody, MM1, to form a sandwich assay for the measurement of PSA-ACT. Reaction rates of the PSA-ACT calibrators, and the analyte concentrations of 15 prostate cancer patient serum samples were compared using the two anti-ACT antibodies. Results showed that ACT 53 gave reaction rates which were ≥52% higher than that for ACT 68. Furthermore, background signal using ACT 68 was significantly higher than that seen with antibody ACT 53. Because antibody

ACT 53 demonstrated a higher signal to noise ratio, it was selected for further analysis.

Standard Curves of Both Bayer Immuno 1 PSA-ACT and ELISA PSA-ACT Assays

[0045]   A standard curve was derived using the six-level PSA-ACT calibrators run on the automated Bayer Immuno 1 PSA-ACT Assay and results are shown in Figure 1A. A standard curve was also derived using the six-level PSA-ACT calibrators run in the manual microplate PSA-ACT Assay and results are shown in Figure 1B. In both cases, the signal was proportional to the concentration of PSA-ACT.

Recovery of PSA-ACT from Mixtures Containing Free PSA and PSA-ACT

[0046]   Five mixtures with various proportions of free and ACT-complexed PSA at free PSA:PSA-ACT ratios of 100:0, 80:20, 50:50, 20:80 and 0:100 were diluted to a total PSA concentration of approximately 10 ng/mL using the same TRIS buffer used to prepare the PSA-ACT calibrators. These samples were run in each of three assays on the Bayer Immuno 1 analyzer: total PSA, free PSA, and PSA-ACT. To measure total and free PSA, the Bayer Immuno 1 system was calibrated with calibrators prepared using free PSA. For the measurement of PSA-ACT, the Bayer Immuno 1 system was calibrated using calibrators prepared with PSA-ACT. The automated Bayer Immuno 1 PSA-ACT Assay used the ACT 53 detection antibody as described above. Results in Figure 2A show that the Bayer Immuno 1 PSA-ACT assay measures PSA-ACT accurately, does not detect free PSA, and has no apparent problem with over-recovery of PSA-ACT as has been reported for previously described assays measuring PSA-ACT.

[0047]   In addition, the specificity of the solid phase microplate-based ELISA PSA-ACT assay was determined to compare with that of the Bayer Immuno 1 microparticle-based PSA-ACT assay. Samples containing approximately 10 µg/mL total PSA with various proportions of free PSA and PSA-ACT as described above, were tested in the solid phase microplate-based ELISA PSA-ACT assay. Results shown in Fig. 2B demonstrate that recovery of PSA-ACT was not linear with PSA-ACT concentration, and that PSA-ACT measurement was inaccurate in this assay, particularly at low concentrations of PSA-ACT. This is in marked contrast to the recovery of PSA-ACT in the microparticle-based assay. As an example, the 80:20 sample should contain 20% PSA-ACT and should read 2 ng/mL in an immunoassay. Recovery of this sample in the microparticle-based assay was approximately 2 ng/mL, as expected. In contrast, the microplate-based ELISA measured the concentration of PSA-ACT in this sample as 4 ng/mL, which is 100% higher than expected. Repeated manipulations of the microplate-based ELISA assay did not correct

this over-recovery problem.

Specificity of the Bayer Immuno 1 PSA-ACT Assay

[0048] Both free PSA and PSA-ACT at PSA concentrations of 0, 2, 10, 25, 50 and 100 ng/mL were tested as unknown samples using the automated Bayer Immuno 1 PSA-ACT Assay. Results in Figure 3 show that no free PSA was detected by the Bayer Immuno 1 PSA-ACT assay. However, the recovery of free PSA was linear with expected values as determined by the Bayer Immuno 1 fPSA assay. These results demonstrate that the Bayer Immuno 1 PSA-ACT Assay is specific for PSA-ACT with no detectable reactivity with free PSA.

PSA-ACT Spiked Recovery from Patient Serum Samples.

[0049] PSA-ACT at three concentrations of 0, 18 and 61 ng/mL was spiked into three prostate cancer patient serum samples. The PSA-ACT concentrations in these three patient sera were measured using the Bayer Immuno 1 PSA-ACT assay, and compared to values obtained with the Bayer Immuno 1 total PSA assay which is equimolar and accurately measures both free and complexed PSA. Data shown in Fig. 4 indicate an average of 98% recovery of the spiked PSA-ACT for all 3 samples in the PSA-ACT Assay. Because free PSA is measurable in the total PSA Assay, but not in the PSA-ACT assay, the 2% difference in quantitation by the Bayer Immuno 1 total PSA Assay versus the Bayer Immuno 1 PSA-ACT Assay may be due to dissociation of free PSA from the PSA-ACT complex.

Accuracy of PSA-ACT Measurement

[0050] The mean values for (free PSA + PSA-ACT)/total PSA using the Bayer Immuno 1 PSA-ACT assay were 96% in prostate cancer patient sera, 102.7% from BPH patient sera and 103% from normal age-matched serum samples. This indicates that no over-recovery of PSA-ACT in sera was obtained using the Bayer Immuno 1 PSA-ACT assay. In contrast, recoveries using the microplate-based ELISA PSA-ACT assay were approximately 171%, 135% and 164% for the same patient sera. These data confirm results published by other laboratories that microtiter plate ELISA measurement of PSA-ACT yielded higher than expected results (13, 14,16).

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Claims

1. A method for determining PSA-ACT in a blood sample, by immunoassay, characterized in that:

    (a) the blood sample is mixed with a first antibody reagent specific for binding to PSA and a second antibody reagent specific for binding to ACT, to form a reaction mixture containing immobilized and labeled immunocomplexes of PSA-ACT, said immunocomplexes comprising PSA-ACT bound to said first and second antibody reagents wherein one of said first and second antibody reagents is immobilized to microparticles (capture antibody), and the other of said antibody reagents is labeled (detection antibody),
    (b) the microparticles comprising said immobilized immunocomplexes are separated from the remainder of said reaction mixture,
    (c) the separated microparticles are washed, and
    (d) the detection antibody reagent is measured in the washed microparticles and said measurement is related to the concentration of PSA-ACT in the blood sample.

2. The method of claim 1 wherein said microparticles have a mean diameter of between about 0.01 microns and about 100 microns, preferably between about 1 micron and about 50 microns.

3. The method of claim 1 wherein the washing step (c) comprises mixing the separated microparticles with a first wash liquid, separating the microparticles from the first wash liquid, mixing the microparticles separated from the first wash liquid with a second wash liquid, and separating the microparticles from the second wash liquid.

4. The method of claim 3 wherein the wash and separation steps are performed by an automated analyzer.

5. The method of any one of claims 1 to 4 wherein the first and second antibody reagents comprise monoclonal antibodies and/or the microparticles are magnetically responsive.

6. The method of claim 1 wherein said reaction mixture containing immobilized and labeled immunocomplexes of PSA-ACT is formed by mixing the blood sample with said capture and detection antibodies, wherein said capture antibody is immobilized, or capable of being immobilized, to said microparticles (capture antibody), and said detection antibody is labeled, or capable of being labeled (detection antibody), the detection antibody prefer-

ably being labeled with an enzyme or chemiluminescent compound.

7. A test kit for use in determining PSA-ACT in a blood sample, comprising:

    (1) a first antibody reagent specific for binding to PSA, and
    (2) a second antibody reagent specific for binding to ACT, wherein one of said first and second antibody reagents is immobilized to, or is capable of being immobilized to, microparticles (capture antibody), and the other of said antibody reagents is labeled, or capable of being labeled (detection antibody).

8. The test kit of claim 7 where said microparticles have a mean diameter of between about 0.01 microns and about 100 microns, preferably between about 1 micron and about 50 microns.

9. The test kit of claim 7 wherein the detection antibody is labeled with an enzyme or chemiluminescent compound.

10. The test kit of any one of claims 7 to 9 wherein the first and second antibody reagents comprise monoclonal antibodies, and/or the microparticles are magnetically responsive.
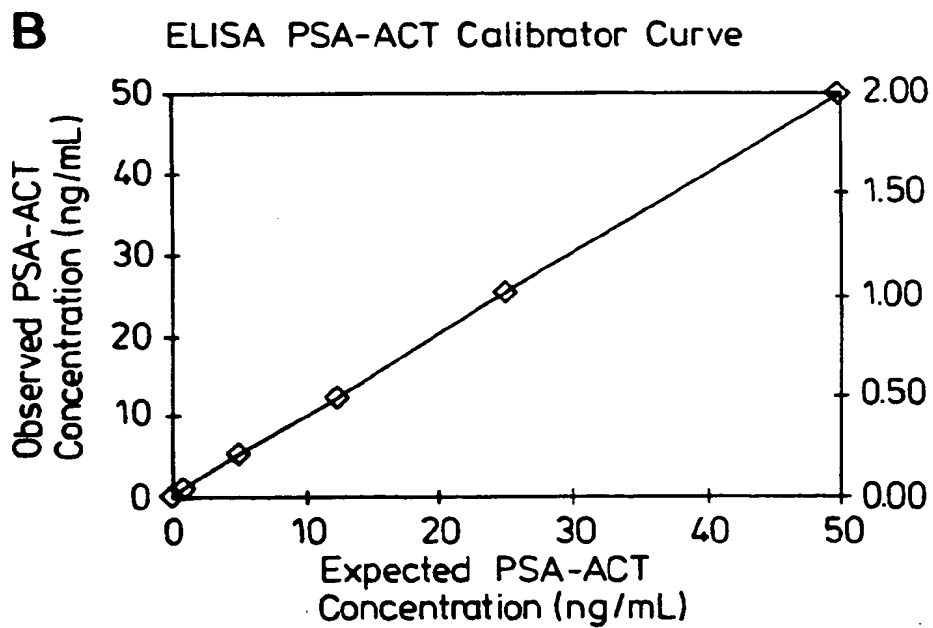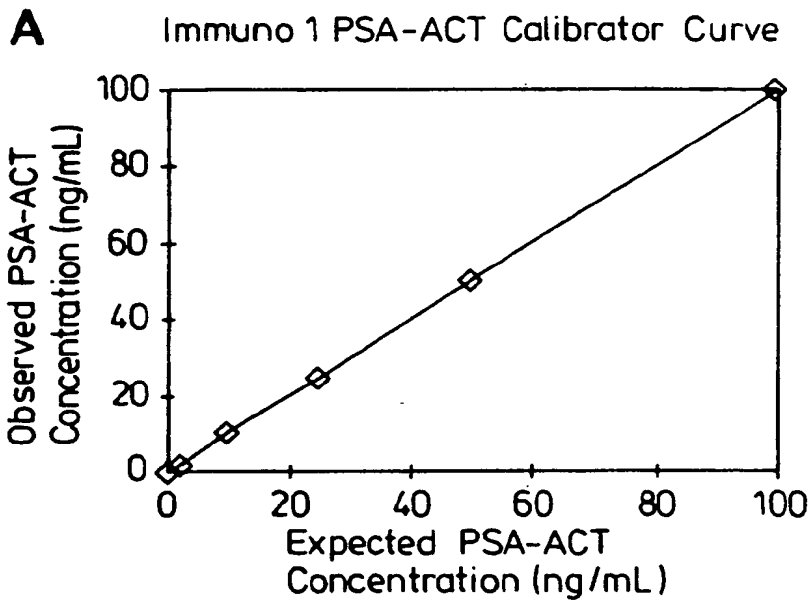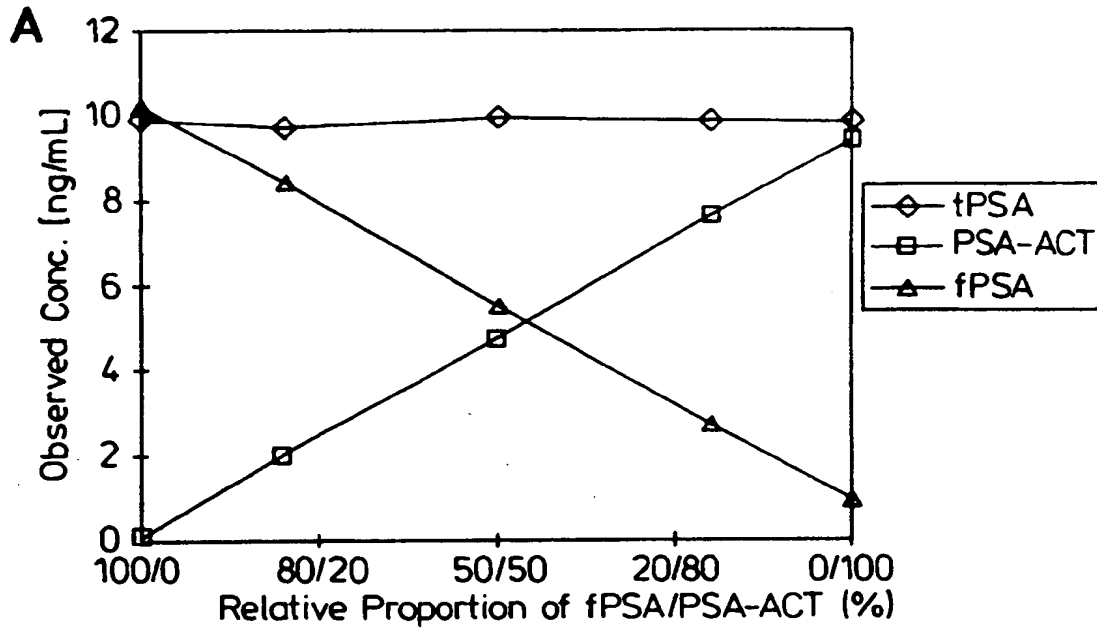
Fig. 1

A   Immuno 1 PSA-ACT Calibrator Curve

B   ELISA PSA-ACT Calibrator Curve

Fig. 2

A

B PSA-ACT Recovery from 5 Mixtures at 10ng/mL
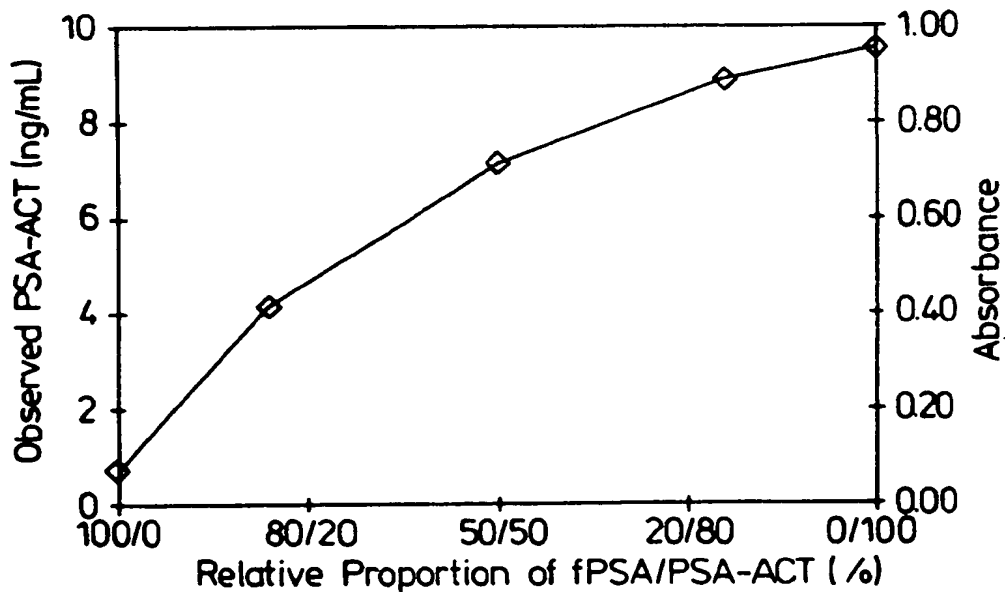
Fig. 3
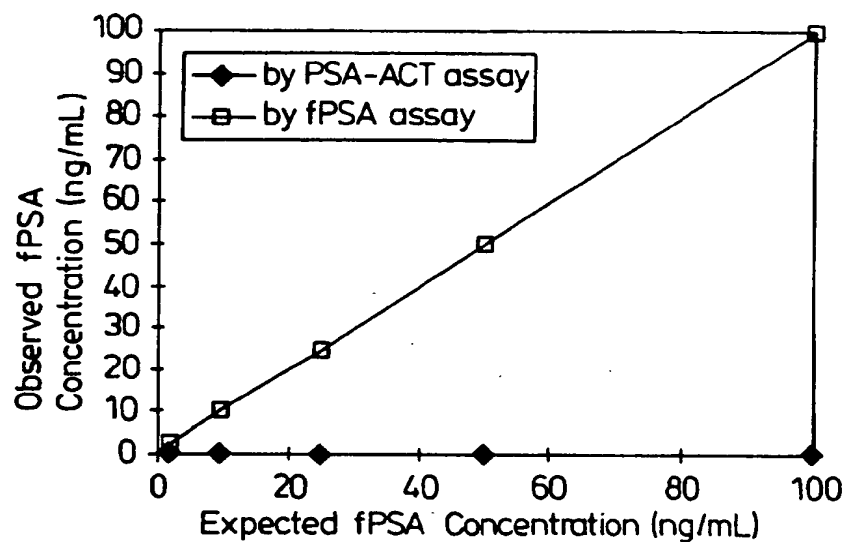
Fig. 4

[PSA-ACT] Expected* (ng/mL)			[PSA-ACT] Observed (% Recovery)		
Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
6.8	20.5	12.4	6.8 (100)	20.5 (100)	12.4 (100)
25.0	38.7	30.6	24.8 (99.2)	37.4 (96.6)	29.5 (96.4)
68.1	81.8	73.7	65.5 (96.1)	78.7 (96.6)	75.2 (102)
% Average			98.4	97.6	99.5
(+/-SD)			2.1	2.1	2.8

*: Expected Concentration of PSA-ACT reflects the concentration plus the measured endogenous concentration.

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EUROPEAN SEARCH REPORT

Application Number

EP 98 11 3378

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	CHICHIBU K ET AL: "SPECIFIC ENZYME IMMUNOASSAY FOR PROSTATE SPECIFIC ANTIGEN-ALPHA1-ANTICHYMOTRYPSIN COMPLEX (PSA-ACT) IN CLINICAL APPLICATIONS" JOURNAL OF UROLOGY, vol. 155, no. 5, May 1996, page 1 XP002061770 * the whole document *	1-10	G01N33/574 G01N33/543
D,X	WANG T I ET AL: "DEVELOPMENT OF MONOCLONAL ANTIBODIES SPECIFIC FOR THE PSA-ACT COMPLEX AND THEIR INCORPORATION INTO AN IMMUNOASSAY" CLINICAL CHEMISTRY, vol. 43, no. SUPPL. 06, 24 July 1997, page S225 XP002061771 * the whole document *	1-10	
D,X	WO 95 18381 A (ABBOTT LAB) 6 July 1995 * the whole document *	1-10	
D,X	ZHOU Z ET AL: "TECHNICON IMMUNO 1 PSA ASSAY MEASURES BOTH FREE AND ALPHA-1-ANTICHYMOTRYPSIN-COMPLEXED PROSTATE-SPECIFIC ANTIGEN ON AN EQUIMOLAR BASIS" JOURNAL OF CLINICAL LABORATORY ANALYSIS, vol. 10, no. 3, 1996, pages 155-159, XP000674624 * the whole document *	1-10	**TECHNICAL FIELDS SEARCHED (Int.Cl.6)** G01N
D,X	DE 43 22 342 A (GIF GES FUER IMMUNOLOGISCHE FO) 9 February 1995 * page 2-5; claims 1-13 *	1-10	
P,X	EP 0 838 680 A (BAYER AG) 29 April 1998 * claims 1-27 *	1-10	

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	13 October 1998	Gundlach, B

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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EUROPEAN SEARCH REPORT

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EP 98 11 3378

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
P,X	WO 98 22509 A (BECKMAN INSTRUMENTS INC) 28 May 1998 * abstract; claims 1-15 *	1-10	

TECHNICAL FIELDS
SEARCHED (Int.Cl.6)

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